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<p>(54) Title: SOLID PHASE ASSEMBLY AND RECONSTRUCTION OF BIOPOLYMERS</p> <p>(57) Abstract</p> <p>The present invention concerns the construction of solid phase assembly of biopolymers through assembly of shorter biopolymer sequences, for example, assembly of genes from oligonucleotides, polypeptides from oligopeptides, and polysaccharides from oligosaccharides. The present invention also relates to the remodeling, or reconstruction of biopolymers, wherein a section of the biopolymer sequence is excised, then replaced by a modified segment.</p>		

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SOLID PHASE ASSEMBLY AND RECONSTRUCTION  
OF BIOPOLYMERS

5

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention concerns the construction of biopolymers through assembly of shorter biopolymer sequences, for example, assembly of genes from oligonucleotides, polypeptides from oligopeptides, and polysaccharides from oligosaccharides. The present invention also relates to the remodeling, or reconstruction of biopolymers, wherein a section of the biopolymer sequence is excised, then replaced by a modified segment.

Background of the Invention

DNA is the chemical substance that makes up the genomes of most life forms. Two properties of DNA that are fundamental to its in vivo function and to the ability of scientists to manipulate it in vitro are that (i) DNA is composed of four different subunits ("bases"), adenine (A), guanine (G), cytosine (C) and thymine (T), linked together by a sugar-phosphate backbone to form long polymeric strands, and (ii) two "complementary" strands of DNA come together to form a double helical DNA molecule by specific hydrogen bonded base pairing (A pairs with T and G pairs with C). This specific base pairing plays an important role in chromosomal replication, a process in which the two DNA strands of a chromosome become separated, then a DNA polymerase enzyme uses each strand as a "template" to synthesize a complementary strand which then base pairs with the template strand, thereby resulting in the formation of two chromosomes from one.

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1           Base pairing also functions in the process of  
"transcription," wherein an RNA polymerase enzyme utilizes  
the base pairing properties of one strand of a gene to  
synthesize a "messenger RNA" molecule (a nucleic acid in  
5       which uracil replaces thymine and the sugar is ribose  
instead of 2"-deoxyribose). The messenger RNA is  
subsequently "translated" into protein as directed by the  
genetic code (each 3-base "codon" in the messenger RNA  
specifies a certain amino acid to be incorporated into a  
10      protein product). Thus, for each gene, the coupled  
transcription/translation process results in biosynthesis  
of a protein molecule that contains an amino acid sequence  
that is encoded by the base sequence in the DNA. In turn,  
the amino acid sequence in the protein determines how the  
15      protein folds into a specific structure and how it  
interacts with other molecules in its biochemical  
function, for example, catalysis of a specific chemical  
reaction in the case of an enzyme.

          Finally, base pairing forms the basis of the  
20      "annealing" reaction that is employed in a variety of  
laboratory DNA manipulations: Two separated DNA strands  
will spontaneously pair up to form a duplex structure  
throughout the region(s) of complementarity if, and only  
if, they contain one or more stretches of complementary  
25      base sequence.

          Recent development of techniques for manipulation  
of genes and proteins, which are used extensively in the  
fields of genetic engineering and biotechnology has  
allowed chemical synthesis of DNA molecules of any desired  
30      base sequence, which can be used to alter existing genes  
or create new ones. This capability, a fundamental tool  
in "protein engineering," allows analysis of the  
structure/function relationships within proteins and  
creation of superior enzymes and drugs in the  
35      biotechnology and pharmaceutical industries.

1 Over the past thirty years, methods for chemical  
synthesis of DNA have rapidly developed. Michelson and  
Todd first chemically synthesized a dinucleotide  
containing the correct phosphodiester linkage (Michelson  
5 and Todd, J. Chem. Soc.:2632-2638 (1955)). Khorana  
developed and used the phosphate triester method of DNA  
synthesis to produce a gene encoding transfer RNA  
(Agarwal, et al., Nature (Lond.), 227:27-40, (1970)).  
More efficient phosphate triester DNA synthesis procedures  
10 were subsequently developed (Letsinger and Ogilvie, J. Am.  
Chem. Soc., 89:4801-4803, (1967); Narong et al., Meth.  
Enzymol., 65:610-620 (Letsinger et al., J. Am. Chem. Soc.,  
97:3278-3279 (1975); Beaucage and Caruthers, Tet. Lett.,  
22:1859-1862, (1981)). Efficient utilization of solid  
15 phase supports for chemical synthesis of DNA have been  
disclosed (Matteucci and Caruthers, J. Am. Chem. Soc.,  
103:3185-3191 (1981); Sproat and Bannwarth, Tet. Lett.,  
24:5771 -5774 (1983)).

The use of solid phase supports for chemical  
20 synthesis of DNA contributed most importantly to the  
ability to rapidly and efficiently synthesize DNA  
chemically, because the growing chain is covalently  
attached to an insoluble support, permitting reagents to  
be washed away between chemical steps, thus eliminating  
25 the need to purify the polynucleotide product after each  
addition of monomer. Furthermore, solid phase synthesis  
permits automation of the process, so that each base  
addition (via multistep reaction cycle) can be carried out  
in about ten minutes at room temperature (Smith, American  
30 Biotechnology Laboratory (Dec., 1983); Caruthers, Science,  
230:281-285 (1955)).

It is now possible to construct a duplex DNA  
molecule encoding a protein or portion thereof, and use of  
the synthetic duplex fragment to construct a recombinant  
35 DNA which can be expressed in vivo to obtain a novel gene  
product. However, the widespread application of gene

1 synthesis has been hindered by: (i) the high cost of  
synthesis of all the oligonucleotides needed to assemble  
an average gene (typically \$5,000 to \$20,000); and (ii)  
5 the slow and labor intensive nature of gene assembly from  
synthetic oligonucleotides. Chemical synthesis of DNA  
currently produces polynucleotides up to 100-150 bases in  
length (and at the upper limits the yield is very low).  
The coding portion of the average gene, however, consists  
10 of 1000-base pairs. Thus, in order to assemble a gene, a  
series of overlapping, complementary oligonucleotides must  
be synthesized, then "annealed" together (i.e., mixed  
together and incubated under conditions that favor  
formation of the double helix between complementary  
15 sequences within the two strands). The duplex DNA, which  
contains strand interruptions at alternating positions  
along the two strands, is then converted to a contiguous  
duplex segment, by enzymatic ligation. Only then can the  
duplex DNA be cloned into a vector for subsequent analysis  
and expression (protein production). In practice, the  
20 correct assembly of a gene from a complex mixture of  
oligonucleotides is difficult to achieve in a single  
annealing step, due to formation of a variety of  
undesirable annealing products. A series of laborious  
purification and analytical steps must normally be carried  
25 out before the intact gene is isolated.

Solid phase procedures for chemical synthesis of  
peptides are frequently based on the protocol of  
Merrifield, which has been successfully used for synthesis  
of enzymatically active, 124-residue ribonuclease A (Gutte  
30 and Merrifield, J. Biol. Chem., 246:1922-1941 (1971)).  
This procedure uses polystyrene-divinylbenzene supports,  
t-butyloxy-carbonyl (tbo) amino group protection, and  
DCC-activated condensation with symmetric anhydride  
intermediates, and has been adapted for fully automated  
35 peptide synthesis. Another procedure for chemical  
synthesis of peptides (known as the "Fmoc" procedure)

1 utilizes a composite polyamide-Kieselguhr support  
(superior for continuous flow synthesis), together with  
fluorenylmethoxycarbonyl (Fmoc) amino group protection,  
and N-hydroxybenzotriazole-activated condensation with  
5 pentafluorophenyl ester (PFPE) intermediates or  
symmetrical anhydride intermediates (Auffret and Meade,  
Synthetic Peptides in Biology and Medicine, Alitalo et al.  
(Eds.), Elsevier Science Publishers, Amsterdam, (1985)).

10 As with DNA, chemical synthesis of peptides,  
prior to the present invention was possible for chain  
lengths up to 100-200 residues (with very low yields at  
these upper limits). More typically, peptides of 20-30  
residues are produced. Assembly of peptides into large  
polypeptides is technically feasible, by ordered, stepwise  
15 condensation of peptides via the Fmoc procedure. But  
again, this approach is expensive and requires laborious,  
time consuming purification of products after each block  
condensation reaction.

The high expense of synthesizing the large  
20 numbers of polynucleotides and peptides needed to assemble  
genes and proteins is largely overcome by use of the  
segmented synthesis technology described in U.S. patent  
application Ser. No. 07/000,716, filed Jan 6, 1987),  
whereas the assembly of these biopolymer fragments into  
25 genes and proteins remains cumbersome and time consuming.

Another technology in genetic engineering and  
biotechnology is the use of enzymes to manipulate the  
genetic material in recombinant DNA research. Restriction  
endonucleases (enzymes that recognize and cleave DNA at  
30 specific sequences, 4-8 base pairs in length) are used to  
isolate specific regions of a chromosome, and DNA ligases  
(enzymes which join together fragments of DNA resulting  
from action of restriction enzymes) are used to "clone"  
the specific DNA fragments into extrachromosomal  
35 replicating genomes (plasmids or viral DNAs), known as  
"vectors" Berg, Science 213:296-303 (1981). The resulting

1 recombinant DNA is used to analyze the base sequence of a  
cloned fragment, or to produce large amounts of a protein  
coded for by a cloned gene. As discussed above, a  
5 powerful extension of this technology is the use of  
chemically synthesized duplex DNA fragments in place of a  
naturally occurring "restriction fragment" in formation of  
a recombinant DNA.

Although recombinant DNA technology represents a  
powerful tool in molecular biology research and genetic  
10 engineering, the labor intensive purification steps and/or  
analysis of numerous reaction products are required before  
a desired recombinant DNA product can be isolated.

Direct manipulation of proteins analogous to  
recombinant DNA methods (Offord, Protein Engineering,  
15 1:151-157, (1987)), allows use of specific endopeptidases  
to excise specific segments from proteins, and then to  
replace these by synthetic pieces, chemically different  
from the natural peptides. This "recombinant protein  
technology" also requires laborious purification steps and  
20 analysis of different reaction products in order to  
isolate the desired engineered protein.

Thus, despite the tremendous power of currently  
available genetic engineering techniques, further  
improvements are needed in the speed, efficiency and  
25 economy of biopolymer manipulations. Accordingly, due to  
the shortcomings of the present procedures, there exists a  
need for a process for rapid, low cost, efficient and  
accurate assembly of biopolymers from their subcomponents,  
and for rapid and convenient in vitro remodeling of  
30 biopolymer sequences, whereby isolation of the desired  
engineered biopolymer is achieved with a minimum of  
purification and analytical steps.

#### SUMMARY OF THE INVENTION

35

It is therefore an object of the present  
invention to provide an improved process for assembly of

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1 biopolymers from subcomponents thereof.

Another object of the present invention is to provide a process for more rapid assembly of genes or gene segments by stepwise annealing of synthetic  
5 oligonucleotides.

Yet another object of the present invention is to provide a more cost effective process for assembly of genes or gene segments by stepwise annealing of synthetic oligonucleotides requiring less labor and materials.

10 A further object of the present invention is to provide a more efficient process for assembly of genes or gene segments by stepwise annealing of synthetic oligonucleotides requiring fewer purification and analytical steps.

15 Still another object of the present invention is to provide a more efficient process for assembly of genes or gene segments by stepwise annealing of synthetic oligonucleotides, providing a greater yield of the desired end-product.

20 An additional object of the present invention is to provide a faster, more efficient and less costly process for assembly of peptides into polypeptides.

A further object of the present invention is to provide an improved process for replacement of a specific  
25 segment of a DNA molecule by an analogous, modified segment or by a different segment.

Yet another object of the present invention is to provide an improved process for replacement of a specific segment of a polypeptide molecule by an analogous,  
30 modified segment or by a new, unrelated segment.

Still another object of the present invention is to provide an improved process for deletion of one or more specific segments within a nucleic acid or protein.

An additional object of the present invention is  
35 to provide an improved process for insertion of one or more oligomeric segments at specific locations in a nucleic acid or protein.

1           Thus, in accomplishing the foregoing objects,  
there is provided in accordance with one aspect of the  
present invention, an improved, general procedure for  
construction of biopolymers, comprising the following  
5 steps: (1) attachment of a biopolymer subcomponent to a  
solid phase support; (2) attachment of the next biopolymer  
sequence to one end of the support-bound component; (3)  
washing away of excess, unattached biopolymer sequences  
added in step (2); (4) ordered, stepwise attachment of  
10 oligomeric biopolymer sequences to the free end of the  
support-bound component (by repeated conduct of steps (2)  
and (3)), resulting in assembly of the biopolymer; and (5)  
release on the assembled biopolymer from the support. All  
steps in the foregoing process can be carried out in a  
15 suspension of the support, or alternatively, in a packed  
bed column fitted with porous means at both ends to  
provide a flow-through system. The biopolymer to be  
constructed by this process is chosen from among the group  
consisting of DNA (genes or gene segments), polypeptides  
20 (proteins), polysaccharides, or any other biopolymer  
composed of subsections that can be joined together. The  
"starting" biopolymer component initially attached to the  
support can range widely in length, for example 1-100  
residues, the precise length being a matter of choice, but  
25 the support-bound starting component will typically be  
10-50 residues in length. The nature of the solid phase  
support is a matter of choice, provided that the structure  
of the support does not sterically hinder the assembly of  
the desired high molecular weight biopolymer. The linkage  
30 of "starting" biopolymer component to the solid phase  
support is a matter of choice, readily achievable by one  
skilled in the art, using a variety of prior art methods.  
The nature of the stepwise linkage of oligomeric  
biopolymer segments during the assembly process, as well  
35 as the method of cleavage of final product from the  
support, will depend on the type of biopolymer being

1 constructed, details of which are given in the embodiments described below.

2 In accordance with one specific aspect of the  
3 present invention, there is provided an improved process  
4 for assembly of a gene (or gene fragment) from synthetic  
5 oligonucleotides, comprising the following steps: (1)  
6 attachment of a "starting" oligonucleotide to the solid  
7 phase support, at or near one of its two ends; (2)  
8 addition of a molar excess of the next oligonucleotide in  
9 the gene to be assembled, one end of the added  
10 oligonucleotide being complementary in base sequence to  
11 the free end of the support-bound oligomer, to form a  
12 molecule in which one end of the added oligonucleotide is  
13 base paired with the support-bound oligomer, leaving a  
14 single-stranded tail at the other end of the added  
15 oligomer; (3) washing away of the unannealed free  
16 oligonucleotides; (4) repeated cycles of oligonucleotide  
17 addition/annealing/washing, carried out until the desired  
18 gene or gene fragment has been assembled; and (5) release  
19 of the assembled DNA from the support.  
20

21 In step (1) of this preferred embodiment, the  
22 solid phase support is first derivatized with a  
23 nucleoside, then the "starting" oligonucleotide is  
24 synthesized on the solid phase support, using standard  
25 phosphate triester or phosphite triester procedures, the  
26 linkage of this synthesized oligonucleotide to the support  
27 being retained and utilized in the subsequent gene  
28 assembly. The solid phase support in this embodiment is  
29 preferably nonporous glass beads of small diameter (5-50  
30 micrometers) or small diameter (5-50 micrometers) glass  
31 beads containing large diameter (1000-5000 A) pores.  
32 Derivatization of the glass beads with nucleoside can be  
33 achieved by a variety of prior art methods that are  
34 readily apparent to one skilled in the art. For example,  
35 the 3"-urethane linkage of a nucleoside to the glass via  
36 long chain alkylamine spacer arm (Sproat and Brown, Nucl.

1 Acids Res., 13:2979-2987, (1985)) can be employed to yield  
a solid phase support suitable for synthesis of the  
"starting" oligonucleotide by standard phosphoramidite or  
5 phosphate triester methods. The urethane linkage is  
largely retained during the deprotection of exocyclic  
amino groups, and can subsequently be utilized for solid  
phase gene assembly. Alternatively, solid phase synthesis  
of the "starting" oligonucleotide can proceed via the  
10 phosphoramidite method or phosphate triester method on  
glass beads derivatized with nucleoside via the standard  
3"-O-succinyl linkage, provided that the sequence of the  
"starting" oligonucleotide is chosen to avoid nucleoside  
residues containing exocyclic amino groups, since the  
15 alkaline condition normally required for deprotection of  
exocyclic amino groups would cleave the DNA from the  
support. For employment of the O-succinyl linkage a  
starting oligonucleotide sequence consisting of thymidine  
and inosine residues would be appropriate.

In step (1) of another preferred embodiment  
20 (attachment of the "starting" oligonucleotide to the solid  
phase support) a preformed oligonucleotide is bonded to  
the support at or near one end. In this embodiment the  
nature of the solid phase support and the method of  
linkage between support and "starting" oligonucleotide are  
25 a matter of choice, readily achievable by one skilled in  
the art, using procedures known in the art with the  
qualification that the structure of the solid phase must  
not sterically hinder the assembly of the gene and the  
linkage of the oligonucleotide to the support must  
30 withstand the conditions of stepwise annealing, used for  
subsequent gene assembly. The solid phase support for  
this embodiment of step (1) appropriately comprises  
nonporous latex microspheres derivatized by functional  
groups, such that chemical crosslinking or condensation  
35 can occur between the beads and a reactive group on one  
end of the starting oligonucleotide in the assembly.

1 Again, the linkage of oligonucleotide to the latex  
particles can be achieved by a variety of established  
procedures which would be apparent to one skilled in the  
art. For example, hydrazide-derivatized latex particles  
5 are readily linked to oligonucleotides derivatized at the  
5'-end with aldehyde or carboxylic acid groups as  
described by Kremsky et al., Nucleic Acids Res., 15:2891  
-2909, (1 987)). Alternatively, alkylamine-derivatized  
beads may be linked to alkylamine-derivatized  
10 oligonucleotides, using a bifunctional crosslinking  
reagent such as disuccinimethyl suberate as described by  
Pilch & Czech, J. Biol. Chem. 254:3375-3381 (1979). In  
addition, alkylamine-derivatized latex particles can first  
be linked to avidin or streptavidin by glutaraldehyde  
15 activation such as described by Goodfried et al., Science  
144:1344 (1964), then the first oligonucleotide in the  
assembly, labeled with biotin at its 5'-end, will attach  
to the beads through the well known tight avidin-biotin  
affinity.

20 The determination of whether the 5' or 3' end of  
the starting oligonucleotide (5' or 3') is attached to the  
solid phase support (which dictates the directionality of  
the gene assembly) is entirely a matter of choice, except  
when the linkage of starting oligonucleotide to the  
25 support is more conveniently achieved at one particular  
end of the oligonucleotide.

Step (2) in the gene assembly process (annealing  
of the next oligonucleotide in the desired gene or gene  
fragment) may be carried out under any of the standard  
30 annealing conditions known to those skilled in the art,  
for example, incubation at 50-65° C in the presence of  
0.2-1 M NaCl or KCl, or incubation at 37° C in the  
presence of 0.2-1 M salt plus 50% formamide. The base  
sequence of the oligonucleotides may be chosen to satisfy  
35 the following specifications: (1) The desired base  
sequence of gene is generated by the assembly process; (2)

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1 The extent of complementary overlap (yielding a duplex  
segment holding the added oligonucleotide to the  
support-bound component) may be any length so long as to  
5 provide the required stability of the association is  
provided. In a preferred embodiment the complementary  
overlap sequence will be at least about 10 bases and may  
be up to 50 bases; (iii) The length of protruding  
single-stranded "tail" after annealing is preferably at  
10 least 10 bases (the length yielding stable base pairing  
with the subsequently added oligonucleotide); (iv)  
Oligonucleotide sequences are preferably chosen to avoid  
secondary structure within the oligonucleotides  
(intrastrand base pairing resulting in hairpins), which  
may interfere with annealing of the added oligonucleotide  
15 to the support-bound component; and (v) The sequences are  
chosen to avoid the production of more than one annealing  
product (through a multiplicity of base pairing  
possibilities).

20 Step (3) in the gene assembly (washing away of  
excess, unannealed added oligonucleotide) can conveniently  
be achieved by flow of solvent past the solid phase  
support, for example through a reaction chamber containing  
porous members at both ends. Alternatively, step (3) can  
be accomplished by a series of brief centrifugation/  
25 decanting steps in microcentrifuge tubes.

In step (4) (ordered, stepwise repeat of steps  
(2) and (3) to build up the desired gene or gene fragment)  
of one embodiment of the present invention, the  
oligonucleotides assembled are designed to yield a  
30 completely duplex DNA with strand interruptions at  
positions alternating along both strands. In another  
embodiment, the oligonucleotides are designed for assembly  
of a partially duplex DNA molecule, in which  
single-stranded gaps exist in alternating positions along  
35 both strands. These gaps may be filled in by action of a  
DNA polymerase in vitro.

1           Step (4) may also be carried out by addition of  
several (eg., about 2-5) oligonucleotides in each  
annealing step. Although this procedure potentially  
5           reduces the total number of annealing steps required for  
assembly of the desired gene or gene fragment, care must  
be taken to insure that multiple products of annealing are  
not generated, i.e., that all support-bound assemblies  
generate the identical, desired duplex DNA sequence.

10           Step (5) (release of the assembled gene from the  
support) is carried out by means chosen to be compatible  
with the nature of the linkage of DNA to the support and  
the structure of the assembled DNA. In one preferred  
embodiment the stepwise annealing is carried out with all  
oligonucleotides being 5'-phosphorylated except for one,  
15           such that a completely duplex DNA is formed in which all  
strand interruptions can be sealed by use of DNA ligase,  
except for a single nonligatable strand interruption  
adjacent to the support-linked oligonucleotide. Then the  
contiguous duplex segment may then be removed from the  
20           support by brief heating to 80-100° C. Alternatively, the  
nonligatable strand interruption adjacent to the support  
can be made by leaving a gap of one or more nucleoside  
residues at this position in the assembled DNA.

25           In step 5 of another preferred embodiment,  
appropriate oligonucleotides are selected for the assembly  
such that a duplex DNA segment containing a restriction  
enzyme recognition sequence is generated between the gene  
or gene fragment and the support, such that release of the  
DNA from the support can be conveniently achieved via  
30           cleavage by the restriction endonuclease.

In all embodiments of the gene assembly process,  
the DNA released from the support is conveniently cloned  
into a vector for expression in cells and DNA sequence  
analysis.

35           In accordance with another aspect of the present  
invention, there is provided a process for assembly of

1 polypeptides, comprising the following steps: (1)  
attachment of a peptide to a solid phase support material;  
(2) stepwise end-to-end block condensation or ligation of  
peptides to the initial support-bound peptide, alternating  
5 with washing steps, to construct a longer polypeptide, and  
(3) cleavage of the polypeptide from the support.

In a particularly preferred embodiment, the solid  
phase support comprises small diameter (5-50 micrometers)  
nonporous glass beads to which the first amino acid  
10 residue is covalently attached via a long chain alkylamine  
spacer arm. In another preferred embodiment, the solid  
phase support comprises small diameter (5-50 micrometers)  
glass beads containing pores of large diameter (1000-5000  
A). Both supports serve to avoid steric hindrance during  
15 the assembly of long polypeptides. The peptide can be  
attached to the supports after the synthesis of the  
peptide, or alternatively, the glass beads can be first  
derivatized with an amino acid residue, then used for  
solid phase peptide synthesis to create a support-bound  
20 peptide which is subsequently elongated in the assembly  
process. Although the preceding embodiments give examples  
of the kind of solid phase support and the type of linkage  
of peptide to the support which may be utilized, these  
parameters are a matter of choice. One skilled in the art  
25 could devise alternate peptide-linked supports that  
possess the favorable steric properties suitable for  
polypeptide assembly. Peptide-linked nonporous latex  
microspheres may also be used as a solid phase support.

In step (2) of one preferred embodiment of the  
30 solid phase polypeptide assembly (stepwise block  
condensation) of the present invention, the stepwise  
condensation of amino terminus-protected peptides onto the  
free amino terminus of a peptide linked to the support via  
its carboxy terminus, is carried out using the standard  
35 Fmoc procedure. In another embodiment, stepwise block  
condensation on the solid phase support is performed



1 chemically, by use of a peptide bond-forming reagent such  
as dichlorophenol, or enzymatically, by "reverse  
proteolysis" (Offord, Protein Engineering, 1:151-157,  
(1987)).

5 In accordance with still another aspect of the  
present invention, there is provide a general procedure  
for remodeling of biopolymer sequences on a solid phase  
support, comprising the following steps: (1) attachment  
10 of a high molecular weight biopolymer at one or more  
positions in the biopolymer sequence to a solid phase  
support; (2) excision of a specific segment of the  
biopolymer; (3) washing away of the cleaving agents and  
excised biopolymer segment; (4) addition of a chemically  
15 synthesized biopolymer sequence or a fragment isolated  
from natural sources and specific insertion of the added  
segment into the biopolymer sequence to replace the  
excised segment; (5) washing away of excess added  
biopolymer segment and bond-reforming agents;, and (6)  
20 cleavage of remodeled biopolymer from the support. The  
foregoing general procedure for biopolymer remodeling can  
also be used to insert or delete biopolymer segments at  
specific positions in the biopolymer sequence.

In step (1) of biopolymer remodeling the nature  
of the solid phase support and means for its attachment to  
25 the support are a matter of choice, depending on the  
structure of biopolymer, and would be readily chosen from  
existing applications by one skilled in the art. For  
example, avidin-coated beads could be used to tightly bind  
biotin-labeled DNA or biotin-labeled protein.  
30 Alternatively, a specific antibody-bound support could be  
used to bind an epitope in a protein or nucleic acid.  
Also, a support-linked oligonucleotide (preferably 20-50  
residues in length) could be used to link a  
single-stranded DNA molecule to the support, via hydrogen  
35 bonded base pairing. In addition, a reversible  
crosslinking agent could be used to connect chemically  
reactive groups in the biopolymer and support.

1           Site-specific cleavage of the biopolymer (step  
2) of solid phase biopolymer remodeling) is preferably  
achieved by enzymatic means, utilizing one or more  
restriction endonucleases in the case of DNA, or specific  
5 endopeptidases in the case of protein. In the specific  
case of single-stranded DNA attached to the support,  
cleavage by restriction endonuclease can be achieved by  
adding oligonucleotides which anneal to the DNA to provide  
short duplex regions containing the enzyme's recognition  
10 sequence. Also, a specific chemical cleavage means (for  
example, cleavage of protein by cyanogen bromide) can also  
be employed in step (2).

          In step (3) of solid phase biopolymer remodeling,  
the cleaving agents and excised biopolymer segment are  
15 washed from the support, preferably by flow of solvent  
past the support-bound biopolymer contained within a  
chamber fitted with porous means at both ends.  
Alternatively, repeated brief centrifugation/decantation  
steps can be used in step (3) for support-bound biopolymer  
20 contained within microcentrifuge tubes.

          In step (4) of solid phase biopolymer remodeling,  
a "replacement" biopolymer segment is added, preferably in  
molar excess over support-bound biopolymer, along with an  
appropriate bond-reforming agent, to achieve replacement  
25 of the biopolymer segment excised in step (2) by the  
segment added in step (4). For example, in remodeling of  
DNA, a restriction fragment isolated from natural sources  
or a chemically synthesized duplex segment containing the  
appropriate termini may be added, and ligated into the  
30 position previously occupied by the segment excised in  
step (2), by the action of DNA ligase. In the case of  
protein, replacement of the excised segment by an added  
peptide can be achieved enzymatically, by "reverse  
proteolysis" catalyzed by specific endopeptidases under  
reaction conditions such as disclosed in Offord, Protein  
35 Engineering, 1:151-157, (1987)), or can be achieved

1 chemically, by action of a peptide bond-forming agent such  
as dichlorophenol.

Washing away of excess reaction components from  
the support-bound biopolymer (step (5)) may be achieved by  
5 the same means as in step (3).

Cleavage of the remodeled biopolymer from the  
support (step (6)) can be carried out by a variety of  
means that would be apparent to one skilled in the art,  
the method of choice depending on the nature of the solid  
10 phase support and biopolymer and the type of linkage  
between them. For example, for biopolymers attached to  
the support via the avidin:SS-biotin affinity, the linkage  
is readily broken by addition of buffer containing 100 mM  
dithiothreitol Shimkus et al., Proc. Natl. Acad. Sci.  
15 (USA) 82:2593-2597 (1985), dissociation of remodeled  
biopolymer from an antibody affinity support can be  
achieved by common protein denaturants, and release of a  
DNA molecule base paired to a support-bound  
oligonucleotide can be achieved by brief heating to  
20 80-100° C.

Further objects, features and advantages of the  
present invention will become apparent from a review of  
the detailed description of the preferred embodiments  
which follows, in view of the drawings, a brief  
25 description of which follows.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic diagram of the solid  
30 phase biopolymer assembly process as applied to  
construction of a gene or gene fragment.

Figure 2 is a schematic diagram of the solid  
phase biopolymer assembly process as applied to  
construction of a polypeptide.

35 Figure 3 is a schematic diagram of the general  
procedure for remodeling of a biopolymer on a solid phase  
support.

1

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention will be described in detail by reference to the drawings. Although the embodiments described herein refer to a few specific uses of the present invention, many variations of the process are also within the scope of the present invention which will be obvious to one skilled in the art.

Figure 1 illustrates the concept of assembly of a gene or gene fragment on a solid phase support. A "starting" oligonucleotide 3 is first attached to a solid phase support 1. As discussed previously, the precise nature of the support 1 and the type of linkage 2 between the starting oligonucleotide and the support are a matter of choice, and are readily known to those of skill in the art. It is essential, however, that the geometry of the solid phase support is such that assembly of the gene is not sterically hindered, as it would be with most currently used solid phase support materials. To satisfy this requirement, a solid phase support consisting of small diameter (5-50 micrometers) nonporous glass beads, or alternatively, macroporous beads (5-50 micrometers in diameter) with very large pores (1000-5000 Å) are recommended for use in solid phase assembly of genes.

The linkage 2 of the starting oligonucleotide to the beads can take a variety of forms, readily known to those of skill in the art. The following examples of suitable linkages are given for illustrative purposes, and it is emphasized that alternative linkages, readily apparent to one skilled in the art, are also within the scope of the present invention.

One suitable linkage 2 to glass beads is the urethane linkage described by Sproat and Brown supra and incorporated herein by reference. The urethane linkage is ideally suited for a synthesis of a starting oligonucleotide of any base sequence prior to gene

1 assembly, since the urethane linkage is more stable than  
the acyl linkages protecting the exocyclic amino groups of  
A, G and C, such that the oligonucleotide will be retained  
on the support under the conditions used for deblocking  
5 the bases.

If the starting oligonucleotide in gene assembly  
is designed to contain a sequence of I (inosine) and T  
(thymidine) nucleosides, then the standard 3'-O-succinyl  
linkage can be used to synthesize the starting  
10 oligonucleotide, because an alkaline base-deblocking step  
(which would hydrolyze the 3'-O-succinyl linkage) would  
not be required after synthesis of oligo(I,T).

Several procedures are available for linkage of a  
presynthesized starting oligonucleotide to the surface of  
15 solid latex microspheres, providing a support-bound  
oligonucleotide suitable for gene assembly. For example,  
the well-known tight avidin-biotin affinity may be  
employed, by covalently linking avidin to small  
alkylamine-derivatized latex beads (0.1-10 microns in  
20 diameter) by the glutaraldehyde activation or other  
methods known in the art, producing avidin-coated beads  
that will bind a 5'-biotin-labeled oligonucleotide.

Latex microspheres may also be covalently  
attached to the starting oligonucleotide for gene assembly  
25 by other methods, including the use of a homobifunctional  
crosslinking agent such as disuccinimethyl suberate to link  
alkylamine-derivatized latex beads with  
5'-alkylamine-derivatized oligonucleotide, linkage of  
hydrazide-derivatized latex beads to a  
30 5'-aldehyde-oligonucleotide or to a  
5'-carboxylate-oligonucleotide and other such linkage  
methods are known in the art (Kremsky et al., Sproat and  
Brown, Shimkus et al., Pilch and Czech, and Goodfriend et  
al., supra, all incorporated herein by reference.

35 The protocol for solid phase gene assembly  
illustrated in Fig. 1 calls for performance of a series of

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1 stepwise annealings, using oligonucleotides 4, 5, 6, 7,  
and n to build up the desired gene or gene  
fragment. The degree of base "overlap" at each annealing  
5 step will preferably result in formation of at least  
twenty base pairs between added oligonucleotide and  
support-bound single-stranded "tail." In the example  
shown in Fig. 1, the starting oligonucleotide is attached  
to the support via its 3"-end, and is  
10 non-phosphorylated. Oligonucleotides added in the  
stepwise annealing reactions are 5"-phosphorylated and  
designed to form a fully double-stranded assembled DNA  
(containing no single-stranded "gaps"), in which the  
strand interruptions (5'-phosphate adjacent to 3'-OH) in  
15 one strand are located at approximately the midpoint of  
the oligonucleotides comprising the other strand. Under  
these conditions, the "nicks" can be enzymatically  
sealed by action of DNA ligase, prior to release of the  
assembled gene from the support.

The stepwise annealing in gene assembly is  
20 preferably carried out in a small volume (eg., 0.02-0.10  
ml), with the solid phase support kept in suspension by  
gentle agitation (except with submicron latex particles,  
which are kept in suspension by Brownian motion). The  
quantity of starting oligonucleotide attached to the  
25 support can vary widely, for example, 0.01-1.0 micromoles  
per gram of beads. At such a "loading capacity" of the  
beads, essentially quantitative stepwise annealing would  
occur within a few minutes under the following reaction  
conditions (0.10 ml annealing volume): 50 mM potassium or  
30 sodium phosphate, pH 7.5, 400 mM KCl or NaCl, 0.1 -1.0  
nanomole of support-bound oligonucleotide, 0.2-2.0  
nanomole of added oligonucleotide, 50-60° C.  
Alternatively, an identical reaction mixture, containing  
addition of 50% formamide, could be incubated at 37° C.  
35 Under the foregoing conditions the concentration of  
annealing DNA (assuming 20 base pair overlap) is 20-200

1 micrograms per ml. The quantity of each added  
oligonucleotide in the gene assembly is very low,  
accommodating the use of inexpensive methods of  
oligonucleotide synthesis that provide low yields of  
5 purified product.

The stepwise annealing of one oligonucleotide at  
a time is recommended, to insure that annealing occurs  
specifically and quantitatively. However, it is possible  
that the procedure illustrated in Fig. 1 could be  
10 successfully adapted to the addition of several  
oligonucleotides at a time, thereby requiring fewer steps  
to assemble a gene. However, even with individual  
annealings, a 1000 base pair gene could be assembled  
within six hours, assuming assembly of fifty 40mers, five  
15 minute annealing time and two minute washing time.

The washing step carried out after each annealing  
reaction, which removes excess unannealed  
oligonucleotides, thus assuring formation of the desired  
annealing product in each cycle, is preferably carried out  
20 by flow of solvent (eg., annealing buffer) past the  
support, which may be provided for by housing the solid  
phase support within a reaction chamber having porous  
means at both ends such as that disclosed in U.S. Patent  
application Serial No. 000,716.

25 Alternatively, 2-3 brief centrifugation/  
decantation steps may be carried out (with support held  
within a microcentrifuge tube) to achieve satisfactory  
washing.

Obviously, in order to obtain the correct  
30 assembly and structure of a gene it is critical that the  
oligonucleotides added at each step be homogeneous.

After the completion of the gene assembly the DNA  
product must be released from the support. In the example  
shown in Fig. 1, this is simply achieved by a brief  
35 heating step (80-90° C), which denatures the short duplex  
section holding the assembled gene to the support, without

1 causing complete denaturation of the long assembled duplex  
DNA (the latter having been converted to contiguous long  
strands by action of DNA ligase). The unsealed strand  
5 interruption at the beginning of the assembled gene (at  
the junction of oligonucleotides 3 and 5 in Fig. 1,  
resulting from the absence of a 5'-phosphate on the  
support-bound starting oligonucleotide) could also be  
arranged by formation of a nonligatable "gap" of at least  
one base at this position.

10 Alternatively, the assembled gene or gene  
fragment could conveniently be released from the support  
by action of a restriction endonuclease, provided that its  
recognition sequence were designed into the duplex DNA  
near the support (eg., within the duplex segment formed by  
15 oligonucleotide 4 in Fig. 1). In the latter case,  
5'-phosphorylation of the oligonucleotides used to  
assemble the gene may be optional.

As illustrated in Fig. 1, the released gene (or  
gene fragment) 9 may be used for any purpose, i.e., it may  
20 be subsequently cloned into a vector for sequence  
analysis, expression (production of protein encoded by the  
gene), etc.

To achieve maximal benefit from the gene  
synthesis and/or assembly of the present invention, the  
25 base sequence of the oligonucleotides to be assembled  
should be carefully planned, with the following  
considerations in mind: (1) The sequences should be  
designed to avoid formation of hairpins of four or more  
base pairs within the oligonucleotides, which may  
30 interfere with efficient intermolecular base pairing  
during the annealing steps. (2) Sequences that are  
commonly associated with poor coupling efficiency during  
the chemical synthesis (such as four or more consecutive G  
residues) should be avoided. (3) Sequences that introduce  
35 "rare codons" into a gene should be avoided, if possible,  
if the aim is to achieve high levels of gene expression.



1 "Rare codons" are those nucleotide sequences which are  
rarely found in nature and thus may not be properly  
translated in some hosts. (4) Oligonucleotides may be  
designed to generate unique restriction sites within the  
5 assembled gene to facilitate subsequent manipulations by  
recombinant DNA techniques. For example, if mutations are  
found at intervals within a chemically synthesized gene,  
the existence of unique restriction sites permits cleavage  
of individual cloned genes with restriction endonucleases,  
10 and recombination to form the desired mutant-free gene.

The length of a duplex DNA that may be assembled  
in the manner illustrated in Fig. 1 ranges widely, from  
less than a hundred base pairs up to thousands of base  
pairs. Because labor-intensive purification and analysis  
15 of intermediates in the gene assembly are avoided by use  
of the solid phase approach, time and expense associated  
with gene construction are greatly reduced by use of the  
present invention. Furthermore, the high efficiency of  
the process permits the use of very small quantities of  
20 DNA, further reducing the cost of gene synthesis. An  
average size gene may be synthesized, assembled and cloned  
into an expression vector within a period of one week, at  
a total cost for materials and labor of less than \$1,000  
if the segmented DNA synthesis device disclosed and  
25 claimed in U.S. Patent Application No. 000,716, filed Jan.  
6, 1987 is used to synthesize the oligonucleotides at 50  
nanomole scale, and then the present invention were used  
to assemble the gene. The cost of preparing the same gene  
by conventional methods would be \$20,000 to \$50,000 and  
30 would typically require about two months work.

The present invention although exemplified as a  
means for gene assembly, is equally applicable to  
construction of other biopolymers, including polypeptides  
and polysaccharides. The method of the present invention  
35 used for polypeptide assembly is shown in Figure 2. A  
protein molecule could be constructed by ordered, stepwise

1 chemical condensation between the free amino terminus of a  
peptide linked to the support via its carboxy terminus and  
successively added amino terminus-protected peptides,  
5 using standard Fmoc chemical condensation known to those  
of skill in the art.

The present invention may also be applied to  
remodeling of a biopolymer, a multistep process which, if  
carried out in solution by conventional means, frequently  
10 requires time-consuming and labor-intensive purification  
and analytical steps before the desired end product is  
obtained. By performing the same manipulations on a solid  
phase support rather than in solution, the need for  
purification and analysis of intermediate products is  
avoided, thus saving time and labor. The advantage of  
15 carrying out a biopolymer reconstruction on a solid phase  
support is illustrated in Figure 3.

Biopolymer 11 is first attached to a solid phase  
support 1 at one or more positions in the biopolymer  
sequence. As explained previously for the process of  
20 biopolymer assembly, the precise nature of the solid phase  
support and the method of linkage of biopolymer thereto  
are entirely a matter of choice, the only constraint being  
that the structure of the solid phase support must not  
restrict accessibility of reaction components to the  
25 biopolymer. Suitable solid phase supports, types of  
linkages, means for washing away reaction components and  
means for ultimate release of biopolymers from the  
supports such as those described previously for biopolymer  
assembly may be utilized.

30 The support-bound biopolymer 11 (for example, a  
double-stranded plasmid DNA) is treated with at least one  
agent (for example, restriction endonuclease(s)) to  
produce cleavage at one or more specific sites 13 within  
the biopolymer sequence. If the biopolymer is cleaved at  
35 two specific sites, one or more specific fragment(s) 12  
were released (for example, a restriction fragment). The

1 released fragment(s) 12 and cleaving agent(s) are  
conveniently washed away, as described previously for  
biopolymer assembly, then a replacement fragment 14 (for  
example, a restriction fragment or synthetic duplex DNA)  
5 is added, and the bonds are reformed (for example, by DNA  
ligase), producing the remodeled biopolymer 15.

This procedure may be used to produce a deletion  
within the biopolymer, by reforming the bonds after  
removal of the released segment 12, without adding back a  
10 replacement segment.

In addition, this procedure may be used to insert  
an additional biopolymer segment into a specific location  
within the support-bound biopolymer, by cleaving at a  
single site within the biopolymer, and then attaching a  
15 biopolymer segment at this position (for example,  
insertion of a "'foreign'" duplex segment or synthetic  
duplex DNA at a unique restriction site within a cloning  
vector, to produce a recombinant DNA).

The application of the present invention to  
20 recombinant DNA technology is advantageous, because of the  
elimination of time-consuming purification steps that are  
typically carried out in order to remove a released DNA  
segment before replacing it with another sequence.

25

#### EXAMPLE 1

##### Remodeling of Bacteriophage M13 Vector

A specific application of the present invention  
for manipulation of single-stranded circular DNA (such as  
a bacteriophage M13 vector) is now given, with reference  
30 to Fig. 3. A circular, single-stranded phage DNA 11 is  
conveniently attached to the solid phase support via base  
pairing with a support-bound synthetic oligonucleotide  
(20-50 bases in length, complementary to a specific region  
within the single-stranded vector). Next, two synthetic  
35 oligonucleotides (eg., 20mers) are added and allowed to  
anneal with the vector sequence, producing short duplex

1 regions containing the restriction sites 13.

Restriction endonuclease(s) are then used to  
cleave out a segment 12 of the DNA between the restriction  
sites, and the restriction enzyme(s) and released fragment  
5 are washed away. Then a replacement fragment 14  
(containing short duplex regions at the ends, with  
identical termini as in the fragment removed) is added and  
joined to the support-bound DNA by action of DNA ligase.  
When the cleavage of support-bound single-stranded vector  
10 is carried out at a single, unique position, a restriction  
fragment or synthetic DNA will be inserted at this  
position. After these manipulations are performed, the  
DNA can be made completely double-stranded by action of a  
DNA polymerase, and as polymerization proceeds through the  
15 short duplex region connecting the vector to the  
support-bound oligonucleotide, the vector is released from  
the former, by the well-known "'strand displacement"  
phenomenon. Finally, the DNA may be converted to closed  
circular form by action of DNA ligase.

20

#### EXAMPLE 2

Synthesis and assembly of a segment of the E.  
coli lacI gene.

#### Solid Phase Support:

25 The support used for both synthesis of the  
"starting" oligonucleotide and for subsequent gene  
assembly consisted of long chain alkylamine-derivatized  
solid glass beads of 6 micrometer diameter, derivatized  
with 5'-trityl,2'-deoxythymidine to form the 3'-O-urethane  
30 linkage (Sproat and Brown, 1985). The "loading" capacity  
of the support, determined by HPLC analysis following  
release of nucleoside from support by 24 hour treatment at  
55° C in concentrated ammonium hydroxide, was 2.2  
micromoles nucleoside per gram of support.

35

1 Synthesis of Oligonucleotides:

45 mg of support was derivatized by the method described by Sproat and Brown with 0.1 umoles of a first nucleoside (A). A non-phosphorylated oligonucleotide  
5 (3'-AAAAAAAAAAAAAAAAAGCGTCGCACGCT-5') was synthesized on the support by the phosphoramidite method (Beaucage & Caruthers, 1981), using a Milligen 7500 DNA synthesizer. Following the final detritylation step, the support  
10 material was placed into a glass vial and treated with 1.5 ml concentrated ammonium hydroxide at 55° C. for 1 hour to remove the protecting groups from the exocyclic amino groups. The support material was then placed into a 1.5 ml Eppendorf tube and washed five times (by  
15 centrifugation/decantation) with annealing buffer (as specified below).

Annealing Conditions for Gene Assembly:

To a 1.5 ml Eppendorf tube was added 0.5 mg oligonucleotide-support (approx. 1 nmole), 2 nanomole of the 5'-phosphorylated oligonucleotide,  
20

5'-pTTTCGCAGCGTGCGAGCGTGCCCGGGTGGT-3'

and sufficient annealing buffer (50 mM  $\text{KH}_2\text{PO}_4$ , pH 7.5, 400 mM KCl, 1mM EDTA) to bring the volume to 0.10 ml. The  
25 tube was incubated at 55° C. for 5 minutes, with occasional gentle agitation, then the tube was centrifuged for 1 minute in an Eppendorf microcentrifuge and the beads were washed twice with 1 ml annealing buffer.

After the first annealing and washing steps the  
30 product is:

Sup-U-O-3'-AAAAAAAAAAAAAAAAAGCGTCGCACGCT-5'

5'-pTTTCGCAGCGTGCGAGCGTGCCCGGGTGGT

35 The annealing/washing cycle was repeated, using 2 nanomole each of the following 5'-phosphorylated oligonucleotides in succession:

1  
3'-CGCACGGGGCCCACTTGGTCCGGTCGGTp-5' (3)

5'-pGAACCAGGCCAGCCACGTTTCTGCGAAAAC-3' (4)

3'-GCAAAGACGCTTTTGAGCTp-5' (5)

5  
The final product, the lacI gene fragment coupled to the solid support is depicted in Figure 4.

Ligation and Cleavage of Duplex DNA From the Support:

10 The support was washed twice with ligase buffer (50 mM Tris-HCl, pH 7.8, 20mM dithiothreitol, 10mM MgCl<sub>2</sub>, 1mM ATP, 0.05 mg/ml bovine serum albumin), then resuspended in 0.098 ml ligase buffer. Two microliters of DNA ligase was added (New England Biolabs, high specific activity grade). After incubation for 30 minutes at 37° C., with occasional gentle agitation, the support was then washed twice with Ava I buffer (10mM Tris-HCl, pH 8, 50mM NaCl, 10mM MgCl<sub>2</sub>, 5mM 2-mercaptoethanol, 0.1 mg/ml bovine serum albumin), then resuspended in 0.098 ml of this buffer. Two microliters of restriction endonuclease Ava I (New England Biolabs) were added, and the mixture was incubated at 37° C. for 30 minutes to cleave the DNA from the support. The tube was centrifuged and the supernatant was collected. The beads were washed twice with 0.1 ml Ava I buffer, and the DNA in the combined supernatants were ethanol precipitated and dissolved in 0.1 ml of 10mM Tris-HCl, pH 7.5 containing 1mM EDTA.

Cloning and Analysis of the Synthetic Gene Segment:

30 Approx. 1 nmol of the duplex lacI gene fragment prepared above was mixed with 1 nmol of M13-lacI-SAXB which had been previously cleaved with Ava I and passed over a Sepharose 2B column to remove the 40-bp segment of the lacI gene. The DNA was ligated with 4% DNA ligase as described above. The DNA was then transfected into E. coli strain JM107 and progeny phage were plated into E.

1 coli strain PD8. This genetic system provides the  
opportunity to assess the possible generation of mutations  
during the chemical synthesis of the lacI gene fragment  
(mutations are seen as blue plaques in the absence of  
5 inducer). The frequency of mutations in this experiment  
was undetectable over the spontaneous frequency.

The DNA of the semi-synthetic M13-lacI was  
sequenced by the "dideoxy" method and found to contain the  
desired wild-type sequence in the region of the chemical  
10 synthesis. Thus, the DNA duplex synthesized by the method  
of the present invention was identical in both sequence  
and mutation frequency to that of the naturally occurring  
wild-type lacI sequence.

Although Fig. 2 illustrates a general procedure  
15 for biopolymer reconstruction (remodeling), many  
variations of the process, specific for different  
bipolymers and different types of manipulations thereupon,  
will be evident to one skilled in the art and are within  
the scope of the present invention. For example, the  
20 solid phase remodeling process could be used to replace a  
specific segment of a protein with a different, modified  
segment, a solid phase "recombinant protein" technique  
analogous to the solid phase recombinant DNA example  
discussed previously.

25 One skilled in the art will readily appreciate  
that the present invention is well adapted to carry out  
the objects and obtain the ends and advantages mentioned,  
as well as those inherent therein. The components,  
methods, procedures and techniques described herein are  
30 presently representative of the preferred embodiments, are  
intended to be exemplary, and are not intended as  
limitations on the scope of the present invention.  
Changes therein and other uses will occur to those skilled  
in the art which are encompassed within the spirit of the  
35 invention and are defined by the scope of the appended  
claims.

WHAT IS CLAIMED IS:

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- 1           1. A process for assembling a biopolymer from  
oligomeric subcomponents thereof, comprising the steps of:
- (1) attaching one end of a biopolymer  
oligomeric subcomponent to a solid phase support;
- 5           (2) attaching a next oligomeric sequence at  
or near the free end of the support-bound  
component;
- (3) removing excess, unattached oligomeric  
sequences;
- 10           (4) repeating steps 2 and 3 for the ordered,  
stepwise attachment of oligomeric biopolymer  
subcomponents at or near the free end of the  
support-bound component resulting in assembly of  
the biopolymer; and
- 15           (5) releasing the assembled biopolymer from  
said support.

2. A process for assembling a gene from  
synthetic oligonucleotides comprising the steps of:
- 20           (1) attaching a first oligonucleotide to a  
solid phase support at or near a first end of  
said oligonucleotide;
- (2) removing excess, unattached  
oligonucleotide;
- 25           (3) hybridizing a next oligonucleotide  
containing at least 5-100 base pairs  
complementary to the free end of said bound  
oligonucleotide wherein said next oligonucleotide  
contains at least 5-100 base pairs more than said  
complementary sequence;
- 30           (4) removing excess, unattached  
oligonucleotide;
- (5) repeating steps (3) and (4), for the  
ordered, stepwise hybridization of  
oligonucleotide sequences at or near the free end
- 35           of the support-bound component; and

1           (6) releasing the assembled gene or gene fragment  
from the support.

3.    A process as claimed in claim 1, wherein  
5 said biopolymer is selected from the group consisting of  
gene or portion thereof, a genome or portion thereof, a  
ribonucleic acid, a polypeptide, and a polysaccharide.

4.    A process as claimed in claim 1, wherein the  
10 successively added components become noncovalently  
attached to the support-bound biopolymer component by  
noncovalent forces, including hydrogen bonding,  
electrostatic interaction or hydrophobic interaction.

15       5.    A process as claimed in claim 1, wherein the  
successively added components become covalently bonded to  
the support-bound biopolymer by means of chemical or  
enzymatic reaction.

20       6.    A process as claimed in claim 1, wherein  
said solid phase support consists of a nonporous,  
particulate material selected from the group consisting of  
silica (glass), latex, polystyrene and plastic.

25       7.    A process as claimed in claim 1, wherein  
said solid phase support consists of a macroporous  
material containing intramatrix spaces (pores) large  
enough to prevent steric hindrance of biopolymeric  
assembly.

30       8.    A process for remodeling of a biopolymer  
partial substitution or modification, comprising:  
a solid phase support material;  
means for attachment of a biopolymer to said  
35 support material at one or more positions in the  
biopolymer sequence;

1 means for excision of at least one specific  
segment of the support-bound biopolymer, the  
excised segment being washed away, then replaced  
by addition of a modified, replacement segment  
5 which becomes specifically attached to the  
support-bound biopolymer, thus providing for  
segmental substitution of the biopolymer; and  
means for removal of the remodeled  
biopolymer from the support.

10

9. A process as claimed in claim 8, wherein the  
support-bound biopolymer is a double-stranded DNA  
molecule, the means for excision comprises a restriction  
endonuclease, the replacement segment comprising a  
15 double-stranded DNA segment, produced synthetically or  
isolated from natural sources, and the means for rejoining  
of the replacement segment to the support-bound DNA  
comprising DNA ligase.

20

10. A process as claimed in claim 8, wherein the  
support-bound biopolymer is a single-stranded DNA  
molecule, the means for excision comprises addition of one  
or more restriction endonucleases and one or more  
synthetic oligonucleotides which anneal to the  
25 single-stranded DNA to create cleavage sites for action of  
the restriction endonuclease(s), the replacement segment  
comprising a double-stranded DNA segment, produced  
synthetically or isolated from natural sources, and the  
means for rejoining of the replacement segment to the  
support-bound DNA comprising DNA ligase.

30

11. A process as claimed in claim 8, wherein the  
support-bound biopolymer is a polypeptide and the means  
for excision comprises a specific endopeptidase, the  
replacement segment comprises a peptide, produced  
35 synthetically or isolated from natural sources, and

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1 wherein the means for linkage of the added segment to the  
support-bound polypeptide comprises chemical condensation  
or enzymatic ligation.

5           12. A process as claimed in claim 8, wherein  
rejoining of the biopolymer is carried out without  
addition of a replacement segment, resulting in deletion  
of one or more segments from the biopolymer.

10           13. A process as claimed in claim 8, wherein  
cleavage of the biopolymer occurs at a single site, and  
insertion of added biopolymer segment occurs at this site.

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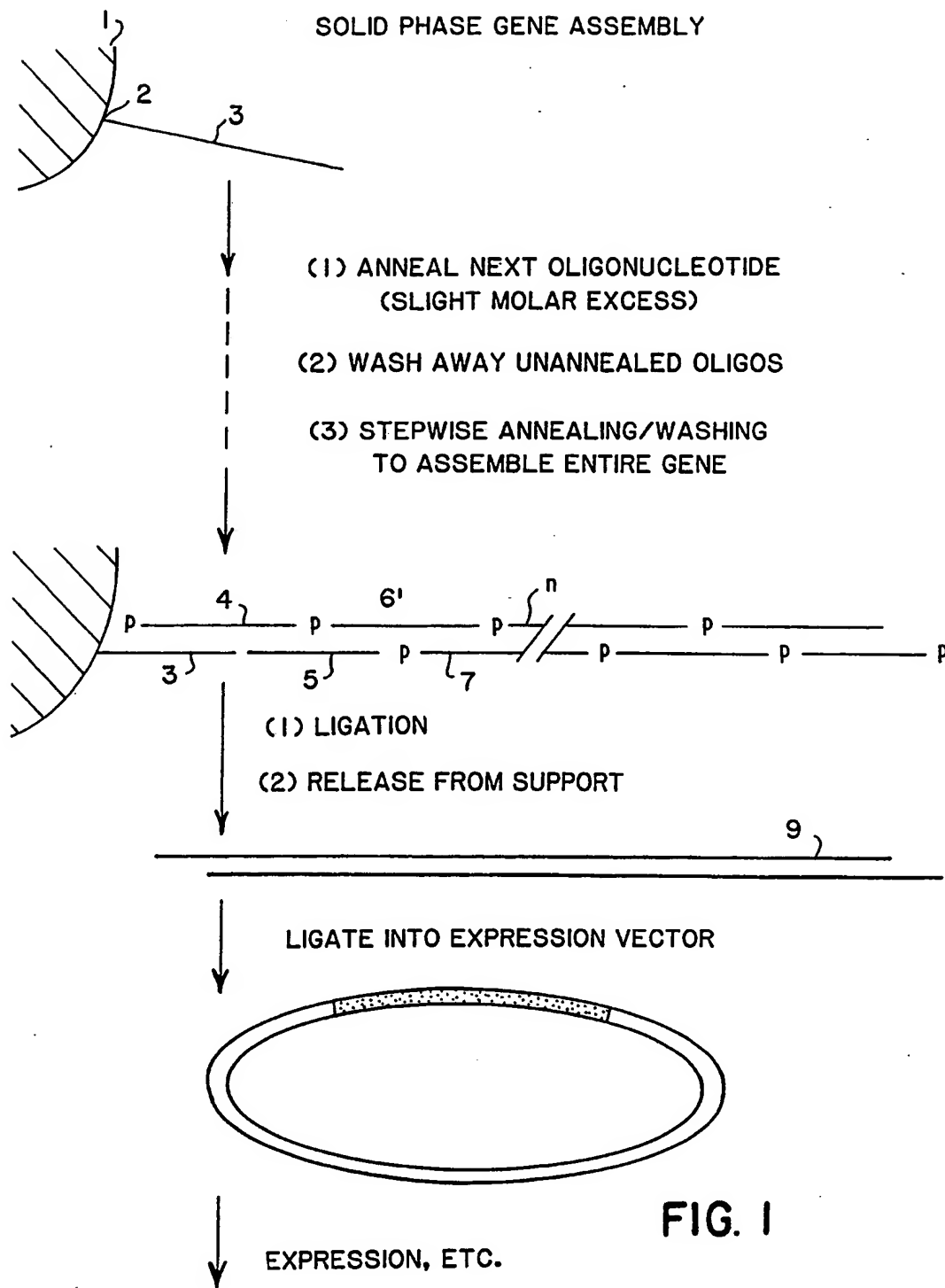
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## SOLID PHASE POLYPEPTIDE ASSEMBLY

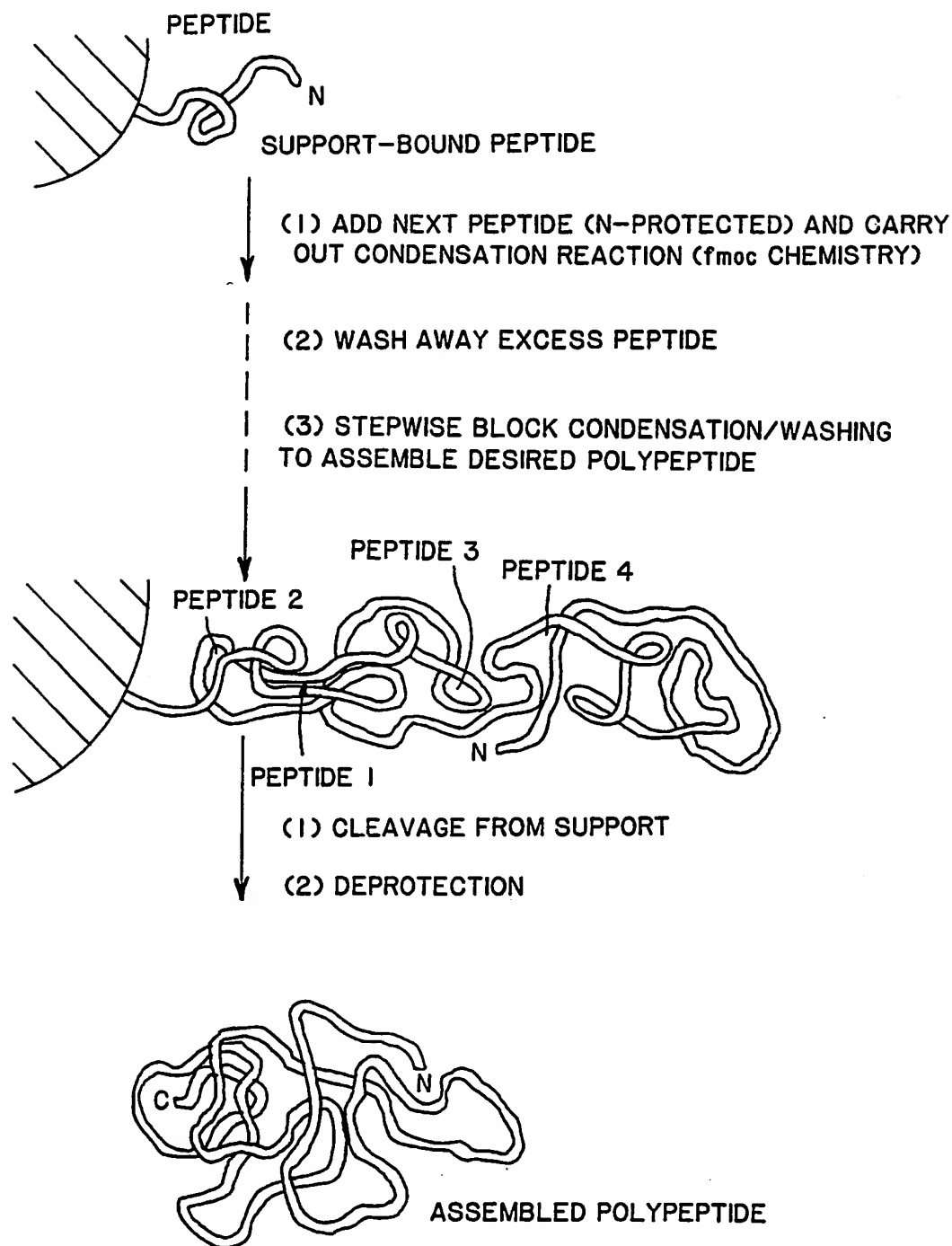


FIG. 2

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## SOLID PHASE REMODELING OF A BIOPOLYMER

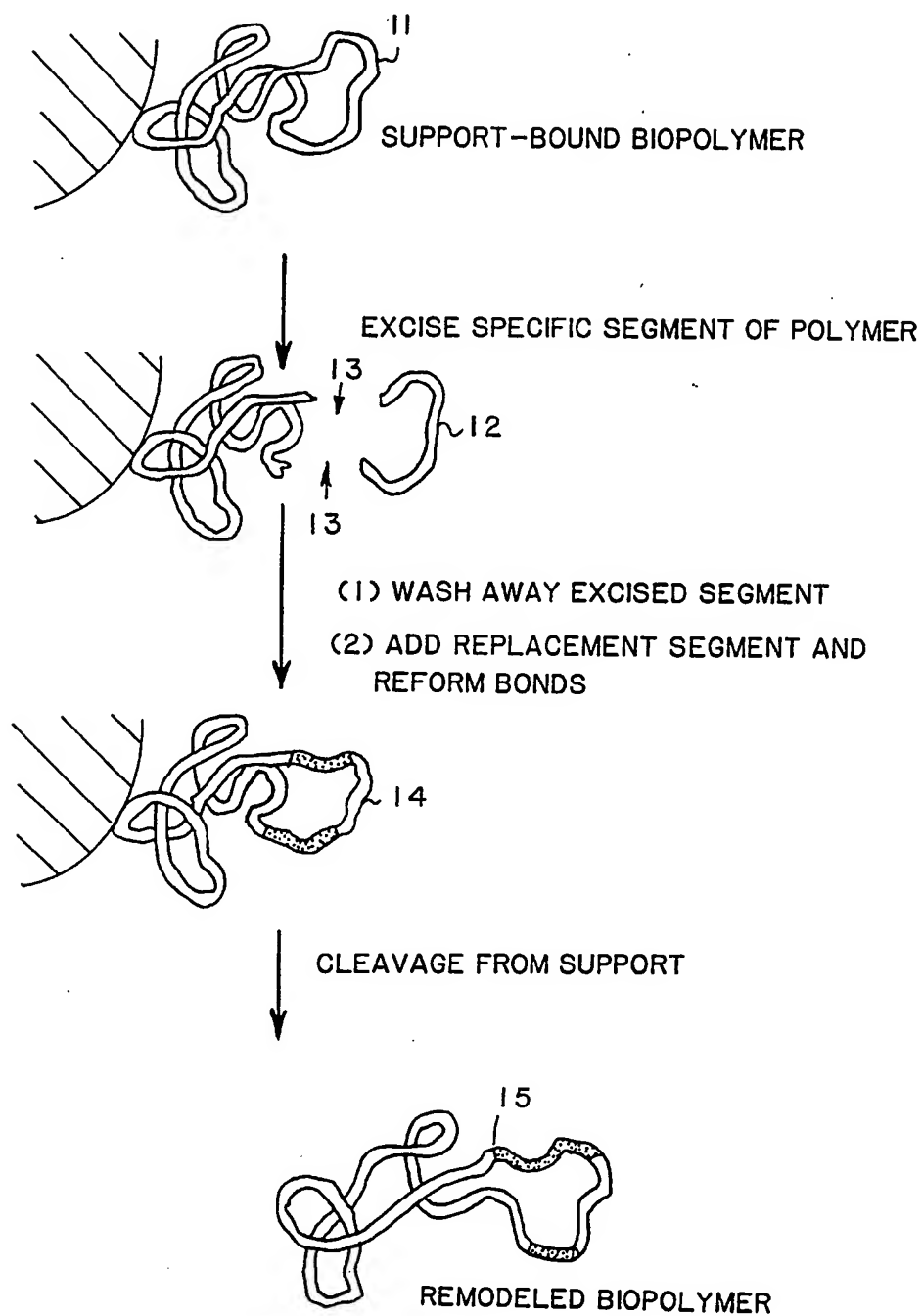


FIG. 3

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**SOLID SUPPORT**

3' -GCAAGACGCTTTTGAGCTp-5' (5)

3' - CGCAGGGGCCACCACTTGTCGGTCGGTp-5' (3)

3'-AAAAAAAAAAAAAAAAAGCGTCGCACGCT-5' (1)

5' -pTTTCGACCGTGGAGCGTGCCCGGTGGT-3' (2)

5' -pGAACCAGGCCACGTTTCTGCGAAAC-3' (4)

**SOLID SUPPORT**

(1)

(3)

(5)

3' -AAAAAAAAAAAAAAAAAGGTGGCAGCGTGGCAGCGGGCCACCACCTTGGTCCGGTCCGTGCAAAAGACGCTTTTCAGCTp-5'

5' -pTTTCCAGCGTGGAGCGTGCCCGGGTGGTGAACCAGGCCACGTTTCTGCAAAAC-3' (2) (4)

(2)

(4)

**FIG. 4**

**SUBSTITUTE SHEET**



# INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US89/02915**

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) 21 to both National Classification and IPC: <b>IPC(4): C12Q 01/68; C12P 21/00; C12P 19/34; G01N 33/00; C07K 1/04</b> <b>U.S. Cl.: 435/6; 435/68; 435/91; 436/86; 436/94; 530/334;</b> <b>SEE ATTACHMENT</b>		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
U.S.	435/ 6, 68, 91, 172.5 436/ 86, 94 530/ 334 536/ 27	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
CHEMICAL ABSTRACT DATA BASE (CA): 1967-1989; BIOSIS DATA BASE: 1969-1989; KEYWORDS: solid phase, gene, oligonucleotide, assembly, synthesis, protein, biopolymer, polypeptide, polysaccharide, muta?		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b>		
Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	NUCLEIC ACIDS RESEARCH, Volume 15, issued 1987, August, (HOSTOMSKY ET AL.), "Solid-Phase Assembly of Cow Colostrum Trypsin Inhibitor Gene", See pages 4849-4856 particularly the abstract.	1-7
Y	ANNUAL REVIEW OF GENETICS, Volume 15, issued 1981, November, (D. SHORTLE), "Directed Mutagenesis", See pages 265-294 particularly pages 266, 270 and 271	8-10 and 12-13
Y	JOURNAL OF ORGANIC CHEMISTRY, Volume 48, issued 1983, March, (NAKAGAWA ET AL.), "Polymer-Bound Oxime: Application to the Synthesis of a Peptide Model for Plasma Apolipoprotein A-I", See pages 678-685 particularly the abstract.	8-10 and 12-13
X	CHEMICAL ABSTRACTS, Volume 107, no. 21, issued 1987, November, (LYLE ET AL.), "Chemical Synthesis of Rat Atrial Natriuretic Factor by Fragment Assembly on a Solid Support", See page 222, column 1, the abstract no. 198913u, J. Org. Chem., 1987, 52(17), 3752-9 (Eng.).	1-8 and 11
<p><sup>*</sup> Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
11 SEPTEMBER 1989		26 OCT 1989
International Searching Authority ISA/US		Signature of Authorized Officer <i>Richard C. Peet</i> RICHARD C. PEET

PCT/US89/02915

I. CLASSIFICATION AND SUBJECT MATTER (CONTINUED)

IPC(4): C12N 15/00; C07H 15/12

U.S.C1.: 435/172.3; 536/27